**K-ras and p53 Gene Mutations in Pancreatic Cancer: Ductal and Nonductal Tumors Progress through Different Genetic Lesions**

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**ABSTRACT**

We studied K-ras and p53 gene mutations in a panel of 57 primary pancreatic cancers including ductal and nonductal tumors. DNA was obtained from formalin-fixed, paraffin-embedded material. Target sequences were amplified by polymerase chain reaction and analyzed by denaturing gradient gel electrophoresis and sequencing. Both K-ras and p53 genes were frequently mutated in ductal cancers (25 of 35, 71.4%; 18 of 35, 51.4%, respectively). K-ras mutations were confined to the second position of codon 12 where base transitions and transversions were equally observed. p53 changes were mainly missense mutations. Transitions and transversions were found equally with a prevalence of G:C→A:T changes among transitions. No gene alterations were present in the 6 exocrine nonductal tumors and (with one exception) in the 12 endocrine tumors analyzed. Our results indicate that mutated K-ras and p53 genes can cooperate in the establishment of ductal pancreatic cancers, whereas other genetic events have to be present in nonductal tumors. Moreover, K-ras alterations may represent an early event in ductal tumorigenesis, as suggested both by the high gene mutation frequency and by the presence of mutations in low-grade tumors. On the contrary, p53 gene changes seem to represent an event required for the malignancy progression of ductal tumors from lower to higher grades.

**INTRODUCTION**

Ductal tumor of the exocrine pancreas is the most common (about 90%) and well-studied pancreatic tumor type. It represents the fourth leading cause of death from malignant diseases in Western countries and its incidence appears to be increasing (1, 2). The aggressive nature of the neoplasia, the lack of early detection, and the refractory resistance to treatment contribute to the high mortality rate from the disease.

Progress in understanding the molecular pathology of pancreatic neoplasias has been restrained by the limited availability of biological material. In spite of this fact, genetic abnormalities have been described, involving growth factors and their receptors (3–6), as well as oncogenes (7–11) and tumor suppressor genes (12–17).

Point mutations of the K-ras protooncogene, almost always confined to codon 12, are the most frequent genetic changes (about 50%) and may represent a relatively early event in pancreatic tumorigenesis (7, 8, 18). Abnormally high levels of p53 gene product have been found in 60% of pancreatic tumors (12, 16), suggesting a high mutation frequency of the gene in this neoplasia. In fact, a high p53 expression level results in most cases from a longer half-life of the protein associated with point mutations within the coding part of the gene (19). More recently, both K-ras and p53 alterations have been identified in pancreatic cell lines, suggesting a cooperation effect which may contribute to the malignant phenotype (15).

We studied K-ras and p53 mutations in a panel of 57 primary pancreatic tumors. The sample included tumor types related to the different cell compartments of the pancreas, i.e., acinar, ductal, and endocrine cells. Our aim was to determine the gene mutation frequencies and to evaluate the cooperative effect of K-ras and p53 alterations in pancreatic tumors of different types and with various degrees of anaplasia. We were also interested in determining the p53 mutation pattern in pancreatic cancer. Although p53 mutations are the most common genetic changes in human cancer, their incidence, distribution, and nature show cancer type and tissue specificity (20, 21). Analysis of the gene can, therefore, provide some clues concerning both endogenous cellular mechanisms and exogenous agents related to mutagenic processes acting in different tissues.

Tumor sample DNAs were obtained from formalin-fixed, paraffin-embedded material and analyzed by PCR3-based DGGE and sequencing methods.

**MATERIALS AND METHODS**

**Tissue Sampling and Morphology.** Surgical specimens of 57 pancreatic tumors were collected from the files of the Pathology Departments of the Istituto di Ricovero e Cura a Carattere Scientifico-Ospedale S. Matteo di Pavia, Ospedale di Circolo di Varese, and Istituto di Ricovero e Cura a Carattere Scientifico-Ospedale S. Raffaele of Milan. Two or three representative formalin-fixed, paraffin-embedded blocks from each tumor were available for histological investigation.

The tumors were classified histologically according to the criteria of Cubilla and Fitzgerald (22). The sample included 35 ductal carcinomas (all with metastases) and 18 nonductal tumors (12 clinically nonfunctioning large endocrine tumors, 8 of which with malignancy proven by metastases and 4 with moderate histological atypia but no metastases; 3 acinar cell carcinomas; 1 papillary cystic tumor; 1 serous cystadenoma; and 1 ductulocarcinoma). Two mucinous cystadenocarcinomas and 2 giant cell carcinomas were also examined. Mucinous cystadenocarcinoma and giant cell carcinoma are interpreted as low- and high-grade growths, respectively, of the same ductal cell involved in the histogenesis of ductal carcinoma.

Grading of ductal carcinomas (13 G1, 15 G2, and 7 G3 tumors) was done according to the criteria of Klöppel (23).

We characterized exocrine tumors with immunoperoxidase by using antibodies against pancreatic and gastrointestinal mucin antigens (24). Monoclonal antibody anti-human lipase (Chemicon, Inc., Temecula, CA) and anti-human trypsinogen (Biodesign Int., Kennebunkport, ME) were used to characterize acinar cell carcinomas. None of the 12 endocrine tumors studied was associated with a hyperfunctional syndrome. Such clinically "nonfunctioning" endocrine tumors were investigated with Grimelius’ silver and by immunohistochemical staining for chromogranin A, insulin, glucagon, somatostatin, pancreatic polypeptide, and gastrin as previously reported (25). All tumors gave widespread staining with general endocrine markers. No hormone immunoreactivity was detected in two of the malignant cases, while in the remaining tumors minority populations of glucagon (6 cases), pancreatic polypeptide (6), insulin (3), gastrin (2), or somatostatin (2) cells were detected.

**DNA Preparation.** Sections (5 μm) of formalin-fixed, paraffin-embedded tumor tissue sections were dissected following closely the distribution of tumor tissue in order to ensure that >70% of the remaining section was formed by normal tissue.

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3 The abbreviations used are: PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Glu, glutamic acid; Gly, glycine; His, histidine; Leu, leucine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.
buffer (50 mM KCl-10 mM Tris-HCl, pH 7.5-2.5 mM MgCl$_2$-0.1 mg/ml gelatin-0.45% Nonidet P-40-0.45% Tween-20-500 tig/ml proteinase K). The solution was heated for 15 min at 95°C to inactivate the proteinase K, and then it was centrifuged. The supernatant (5–10 μl) was used directly for PCR amplification.

**PCR Amplification.** The DNA extracted from tissue sections was a suitable template for the amplification of the relatively short target DNA fragments (111–270 base pairs). The sequences of the specific primers utilized for K-ras and for p53 gene amplification are those we previously reported (Refs. 26 and 27, respectively). One member of each primer pair contained a 5’ 40-base pair GC-rich sequence (GC-clamp). GC-clamped primers were used to obtain amplified products suitable for DGGE analysis.

Amplification was performed on 5–10 μl of DNA by using 12.5 pmol of each primer, 1 unit of thermostable DNA polymerase, and 200 μM deoxynucleoside triphosphates in a volume of 50 μl of buffer (50 mM KCl-10 mM Tris-HCl, pH 8.4-2 mM MgCl$_2$-0.2 mg/ml of bovine serum albumin). Thirty to 40 cycles of amplification were performed in a programmable thermal cycler (MJ Research, Inc.). One cycle consisted of: (a) 1 min at 94°C; (b) 1.5 min at the annealing temperature; (c) 2 min at 72°C.

DGGE. The PCR products obtained with the GC-clamped ampliprimers were analyzed by DGGE. The introduction of a GC-clamp into the amplified fragments brings the probability of detecting any base change within the fragment close to 100% (28).

The gel apparatus was essentially as described by Myers et al. (29). The appropriate DGGE conditions for the analysis of K-ras gene exon 1 (26) and for p53 gene exons 5–8 (27) were as we previously reported. In our hands (26) the sensitivity of the PCR-based DGGE method allows the detection of an heterozygous mutation present in 4% of the cells utilized for DNA extraction. Samples showing a variant DGGE pattern were reamplified and reanalyzed by DGGE in independent experiments.

**DNA Sequencing.** Genomic DNAs from samples showing variant DGGE patterns were amplified using the appropriate primers without GC-clamps in a total volume of 100 μl.

The amplification product was purified on a 2% low-melting agarose gel. The specific band was excised from the gel, frozen in liquid nitrogen for a few minutes, and incubated for 15 min at 37°C. This step was repeated 2 or 3 times to recover all of the DNA. The agarose fragment was centrifuged for 10 min, and the supernatant was quantified and directly utilized for sequencing reactions.

Sequencing was performed by the dideoxy procedure (30) using the Circum Ven(exo-) kit (New England Biolabs, Beverly, MA) for cyclic sequencing. Following the manufacturer’s instructions, 10 ng of the template DNA were mixed with 1.2 μl of the primer, 2 μl of Ven(exo-) polymerase, and 2 μl of α-$^3$P-dATP in 16 μl of buffer [10 mM KCI-10 mM (NH$_4$)$_2$SO$_4$-20 mM Tris-HCl, pH 8.8-5 mM MgSO$_4$-0.2% Triton X-100]. Twenty cycles of amplification were performed in a programmable thermal cycler (MJ Research, Inc.), each cycle consisting of 20 s at 95°C, 20 s at the annealing temperature specific for the primer utilized, and 20 s at 70°C. PCR reactions were electrophoresed on 7% polyacrylamide-7 M urea gels for 2–3 h (1800 V). The gels were then washed for 15 min in 10% methanol and 10% acetic acid. Gels were dried and exposed to β-MAX films (Amersham).

### RESULTS

We searched for K-ras and p53 gene mutations in 57 pancreatic tumors by analyzing DNA from formalin-fixed, paraffin-embedded tissue samples. The screening was performed by using PCR, DGGE, and sequencing methods.

**K-ras Gene.** The first exon of the K-ras gene was amplified with specific ampliprimers, and the PCR product was analyzed by DGGE as we previously reported (26). Tumor samples were analyzed in parallel with DNAs from control cell lines homozygous for the wild-type gene or heterozygous for known mutated K-ras alleles. As mutated cell lines we chose those carrying K-ras base substitutions expected to occur frequently in pancreatic carcinomas. Tumor samples showing variant DGGE patterns were classified by simply comparing their DGGE bands with those of control cell lines as previously reported (26). The few samples showing bands different from those of control DNAs were sequenced in order to characterize the K-ras mutations.

All tumor samples carrying a mutated K-ras allele showed four DGGE bands, including the homoduplex one corresponding to the wild-type gene. This indicates that the neoplastic cells were heterozygous for the observed mutation and/or that nonneoplastic cells contributing the normal allele were present in the tissue sections utilized for DNA extraction. Fig. 1 shows patterns from tumors homozygous (sample 7) and heterozygous (samples 2 and 3) for normal and mutated K-ras alleles, respectively.

We observed K-ras gene mutations in 25 of 35 (71.4%) ductal pancreatic tumors. On the contrary, no mutations were observed in the remaining 22 cases, including 2 mucinous cystadenocarcinomas, 2 giant cell carcinomas, 6 nonductal exocrine tumors, and 12 nonfunctioning endocrine tumors.

All mutations occurred at codon 12, with the exception of one case (patient 29) showing a mutation at codon 13. Of these mutations, 21 (13 G—>A and 8 G—>T) occurred at the second position of the codon. One ductal tumor (patient 10) showed two different mutations at codon 12, namely, GGT→TGT (Gly→Cys) and GGT→AGT (Gly→Ser). All of the K-ras base changes observed are reported in the last column of Table 1.

**p53 Gene.** Exons 5–8 of the p53 gene were amplified with specific ampliprimers, and the PCR products were analyzed by DGGE as we previously reported (27). The exons analyzed contain codons of most of the evolutionary conserved amino acids of p53 protein and include several mutation hot spots (20).

We found 20 tumors with a mutated p53 sequence. All of the mutated samples showed four bands including the homoduplex one corresponding to the wild-type p53 allele (Fig. 2).

In total we observed 14 different variant patterns, since few samples shared the same DGGE bands. Samples showing variant DGGE patterns were sequenced, and both sense and antisense strands were analyzed to confirm the nature of mutations. In all of the samples showing a variant pattern, DNA sequencing revealed the presence of a mutation in the coding part of the gene. The presence of the same base substitution was found in samples showing identical DGGE bands. The variant patterns were due to 3 different mutations localized in exon 5, to 1 in exon 6, to 5 in exon 7, and to 5 in exon 8 (Table 1). Of 20 mutations, 18 were found in ductal carcinomas (18 of 35, 51.4%), whereas one of 18 nonductal exocrine or endocrine
**K-ras AND p53 GENE MUTATIONS IN PANCREATIC TUMORS**

Table 1  K-ras and p53 mutations in pancreatic tumors

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histological type</th>
<th>Histological grade</th>
<th>Exon</th>
<th>Codon</th>
<th>Base change</th>
<th>Amino acid substitution</th>
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<td>56</td>
<td>Histology atypical</td>
<td>Exon</td>
<td>Codon</td>
<td>Base change</td>
<td>Amino acid substitution</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>Histology atypical</td>
<td>Exon</td>
<td>Codon</td>
<td>Base change</td>
<td>Amino acid substitution</td>
<td></td>
</tr>
</tbody>
</table>

*D Codon 13.

Tumors (namely, a nonfunctioning endocrine tumor with moderate histological atypia but no metastases) showed a p53 base change. Another mutation was found in a giant cell carcinoma, a type of tumor interpreted as a growth of anaplastic ductal cells.

Of 20 gene alterations, 17 were missense mutations. Two samples (patients 9 and 35) showed the same nonsense mutation in exon 8 and one tumor (patient 39) showed an in frame deletion of 3 base pairs. Sample 9, in addition to a functional mutation in exon 8, showed a silent nucleotide substitution in exon 6 (Fig. 2). This is a polymorphic variant already found in different population samples (31). Of the 19 point mutations, 11 were base transitions, 9 of them being G:C→A:T. All p53 mutations are reported in Table 1, and specific base changes are summarized in Table 2.

**K-ras, p53 mutations and tumor grading of ductal carcinomas are summarized in Table 3.**

**DISCUSSION**

By PCR-based DGGE and sequencing methods we analyzed K-ras and p53 gene mutations in a panel of 57 pancreatic tumors of different...
possible precursors of ductal carcinoma, and even in some mucous
illary neoplasms, which are low-grade malignancies considered as
genetic alterations may have a crucial role in ductal tumorigenesis.

degrees of anaplasia. The presence of K-ras mutations also in low-
mutations in different tissue areas within the same pancreatic tumors.

same tumor cells and the existence of two subpopulations of cells each
discriminate between the presence of two different mutations in the
position of codon 12. Because of the approach we used, we could not
examine a relatively large number of ductal adenocarcinomas as well
and 11 of 25 transversions (9 G→T and 2 G→C). These substitutions
involvement of specific sites.

Kinases AND p53 GENE MUTATIONS IN PANCREATIC TUMORS

Table 2 p53 point mutations in pancreatic tumors

<table>
<thead>
<tr>
<th>From</th>
<th>C</th>
<th>T</th>
<th>G</th>
<th>A</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>T</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td></td>
<td>19</td>
</tr>
</tbody>
</table>

* Listed in coding strand.

types. The use of archival samples for DNA extraction allowed us to
examine a relatively large number of ductal adenocarcinomas as well
as to select rare tumor types among nonductal exocrine tumors, in-
cluding serous cystadenoma, papillary-cystic tumor, acinar cell carci-
noma, and ductuloacinar carcinoma. In addition, endocrine tumors of
proven or suspected malignancy were investigated.

Our data confirm the remarkably high frequency of K-ras mutations
in ductal carcinomas: we found 25 mutated tumors in 35 cases inves-
tigated, which corresponds to a frequency of 71.4%. As expected, the
mutations were almost exclusively confined to the second position of
codon 12 (21 of 25). We observed 14 of 25 base transitions (G→A)
and 11 of 25 transversions (9 G→T and 2 G→C). These substitutions
correspond to conversions from glycine to aspartic acid, valine, and
arginine, respectively.

One sample (patient 10) showed two different mutations at the same
position of codon 12. Because of the approach we used, we could not
discriminate between the presence of two different mutations in the
same tumor cells and the existence of two subpopulations of cells each
carrying a single K-ras mutation. This last possibility would be in
agreement with the finding by Grunewald et al. (32) of different K-ras
mutations in different tissue areas within the same pancreatic tumors.

During the present survey we analyzed ductal tumors with different
degrees of anaplasia. The presence of K-ras mutations also in low-
grade G1 tumors (7 of 13, Table 3), in addition to the high mutation
frequency found in ductal cancers in general, suggests that these
genetic alterations may have a crucial role in ductal tumorigenesis.

K-ras mutations also have been detected (33, 34) in intraductal pap-
illary neoplasms, which are low-grade malignancies considered as
possible precursors of ductal carcinoma, and even in some mucous
cell hyperplasias of pancreases with chronic inflammation (35). These
findings further support the idea (7, 8, 18) that K-ras mutations are an
early event in ductal pancreatic carcinogenesis.

On the other hand, our data clearly demonstrate that K-ras muta-
tions are not involved in the genesis and progression of nonductal
exocrine or endocrine tumors. In fact, no such mutations were found
in the 18 cases we analyzed. In addition, only one of 16 acinar cell
carcinomas investigated by Hoorens et al. (17) showed a K-ras gene
activation.

The p53 gene analysis allowed us to detect mutations in 18 of 35
ductal cancers (51.4%). On the contrary, none of various kinds of
nonductal exocrine tumors and only one of 12 nonfunctioning endo-
crine tumors showed a p53 mutation. Negative findings also were
reported by Yoshimoto et al. (36) for pancreatic endocrine tumors
(mostly benign) and extrapancreatic endocrine tumors. The mutation
frequency we found in ductal cancers (51.4%) is slightly higher than
the 43.7% figure reported by Scarpa et al. (16) using the single-strand
conformation polymorphism technique.

Abnormal levels of the p53 gene product have been found by
immunohistochemical methods in 60% of ductal pancreatic tumors
(12, 16), and the molecular analysis of samples with immunodetect-
able protein confirmed in most cases the presence of point mutations
within the gene. The mutation frequency we found is comparable to
the rate of p53 overexpression observed immunohistochemically. On
the other hand, no p53 protein overexpression has been detected in
acinic cell carcinomas (17).

All of the p53 changes we observed were point mutations, with the
only exception being a 3-base pair deletion detected in a giant cell
carcinoma. Among point mutations only 2 cases produced a stop
codon.

All of the p53 genetic changes were in the highly conserved do-
 mains of the protein or at conserved amino acid residues. Ten of 20
mutations were clustered in exon 8, and 5 of 10 were in codon 273
which is a known mutation hot spot in various human tumors.

Of 19 p53 changes, 11 were base transitions. While transversions
were more heterogeneous, 9 of 11 transitions were of the same
tumors was also observed by other authors (12, 14, 16). On the whole,
the mutation spectrum we found in ductal pancreatic cancer (Table 2)
appears more complex compared to other tumor types (20, 21). The
action of different carcinogens as well as the occurrence of sponta-
neous base substitutions may contribute to this mutation pattern. In
fact, 8 of 9 G:C→A:T transitions occurred at CpG mutation hot spots
(1 at codon 248, 5 at codon 273, and 2 at codon 282). Spontaneously
occurring deamination at methylated CpG sites is a general mecha-
nism accounting for C→T and G→A changes (37) and has been
indicated as an endogenous cause of somatic mutations in p53 gene
(38). However, only 2 of 8 transitions were at CpG sites (codon 175)
in the study by Scarpa et al. (16), who also failed to detect preferential
involvement of specific sites.

The presence of mutations in both K-ras and p53 genes found in 13
ductal cancers (Table 3) suggests a cooperative effect of K-ras and
p53 alterations in ductal pancreatic tumorigenesis. K-ras activation
may represent an early and critical event in such a process (see above).
On the other hand, p53 changes seem to be especially involved in

Table 3 Gene mutations and histological grading in ductal tumors

<table>
<thead>
<tr>
<th>Presence of mutations</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>K-ras only</td>
<td>5</td>
<td>7</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>p53 only</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>K-ras + p53</td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>13</td>
</tr>
</tbody>
</table>

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more severe tumor cell transformation. In fact, all G3 tumors showed p53 mutations (Table 3), suggesting that these may be relevant to malignancy progression to the higher G3 grade. Finally, our work shows a striking difference at the molecular level between ductal cancers and the remaining pancreatic tumors. In fact, both K-ras and p53 mutations were frequent in ductal cancers (71.4 and 51.4%, respectively), whereas no alterations (with the exception of a single case showing a mutated p53) were present in a variety of rare non-ductal tumors we examined. All of the latter tumors involve cell types, either exocrine or endocrine, normally forming pancreatic lobuli, whereas ordinary ductal cancers are essentially made up of cells mimicking mucin-producing cells of extrabulbar ducts (24). Therefore, the above findings suggest a strong specificity of gene mutations with respect to the different epithelial cell types and compartments of the pancreas. Indeed, specific gene mutations of ductal cells might explain why, among the manifold population of epithelial cells forming the pancreas, a single type, normally accounting for a restricted portion (<10%) of the gland, generates a vast majority (approximately 80%) of its tumors. It may be of interest to investigate whether a similar specificity operates in the tumorigenesis of other organs and tissues with multiple epithelial components.

REFERENCES


**K-ras** and **p53** Gene Mutations in Pancreatic Cancer: Ductal and Nonductal Tumors Progress through Different Genetic Lesions

N. S. Pellegata, F. Sessa, B. Renault, et al.


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